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Belinda Perkins

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Rattenhol *et al.*

Group Art Unit: 1649

Serial No.: 09/807,096

Examiner: Hayes, Robert Clinton

Filed: November 19, 2001

Docket No.: 1406/415

Confirmation No.: 2974

For: METHOD FOR OBTAINING ACTIVE BETA-NGF

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DECLARATION OF DR. SUSAN LOREY
PURSUANT TO 37 C.F.R. § 1.132

Commissioner of Patents
Washington, D.C. 20231

Sir:

1. My name is Dr. Susan Lorey, and I am currently the Head of Validation at Scil Proteins GmbH, assignee for the subject U.S. Patent Application Serial No. 09/807,096.

2. A true and accurate copy of my *curriculum vitae*, which evidences my expertise and credentials, is attached herewith and labeled **Exhibit A.**

3. I have had an opportunity to review the above captioned U.S. Patent Application Serial No. 09/807,096.

4. I have also reviewed the following documents: the Final Official Action issued September 18, 2006 by the U.S. Patent and Trademark Office on the above captioned U.S. Patent Application Serial No. 09/807,096; and US.

Patent No. 5,683,894 of Edwards *et al.* (hereinafter "Edwards") cited in the Official Action.

5. Edwards states in Example 4 that murine pro-NGF was produced by infecting mouse L929 fibroblasts grown in Dulbecco's modified Eagle medium with a vaccinia vector encoding murine pro-NGF. The L929 cells were harvested by scraping, washed, and disrupted by sonication or by two cycles of freeze/thaw. The lysates were then cleared by microcentrifugation.

6. The resulting cleared lysate is characterized by the presence of numerous cellular proteins. As such, it is believed that the cleared lysate would not be viewed in the field as being purified at all, let alone substantially purified.

7. As evidence of the crude nature of the lysate produced by the method of Example 4 of Edwards, the method disclosed therein, absent the virus infection step, was replicated by me as follows. Mouse L929 cells were grown in medium with 10% fetal bovine serum at 37°C. The cells were harvested and pelleted by centrifugation. The cell pellet was washed twice using 10 ml PBS. Afterwards, the pellet was resuspended in 50 mM NaCl/100 mM Tris (pH 7.6), and disrupted by sonication. The lysate was cleared of particulate material by centrifugation for 15 minutes at 4°C. A sample of the crude cell extract was loaded onto an SDS-PAGE.

8. **Exhibit B** is a true and accurate reproduction of a stained SDS-PAGE gel depicting the proteins present in a crude murine L929 cell lysate that was prepared by the method disclosed in Example 4 of Edwards (excepting the virus infection step) in lane 2. **Exhibit B** demonstrates that a clarified cell extract produced by this method is characterized by the presence of numerous contaminating host cell proteins, which correspond to the bands present in lane 2 of **Exhibit B.**

9. This is in contrast to the preparations disclosed in the above captioned U.S. Patent Application Serial No. 09/807,096. Unlike the contaminated extracts of Edwards, the purified pro-NGF inclusion body pellets of the above captioned U.S. Patent Application Serial No. 09/807,096 are characterized by one prominent band when analyzed by SDS-PAGE as shown in

Figure 2 of the above captioned U.S. Patent Application Serial No. 09/807,096, a true and accurate copy of which is reproduced as **EXHIBIT C**.

10. In **EXHIBIT C**, lane U corresponds to a crude extract prior to induction, lane I corresponds to a crude extract after four hours of induction, lane P corresponds to the protein present in the purified inclusion body pellet, and lane S corresponds to the soluble supernatant.

11. Lanes U and I of **EXHIBIT C** show a degree of host cell protein contamination in crude lysates that are similar to those seen in lane 2 of **EXHIBIT B** and would thus be expected to be seen in SDS-PAGE analysis of the clarified crude extracts that be obtained by the method of Example 4 of Edwards. In each case, crude host cell lysates would be characterized by the presence of numerous contaminating host cell proteins.

12. As can be seen by comparing lanes U and I to lane P of **EXHIBIT C**, lane P is substantially purified, whereas one of ordinary skill in the field of protein chemistry would recognize lanes U and I to represent a crude, unpurified host cell extract.

13. The pro-NGF present in the purified inclusion body pellet (*i.e.*, lane P of **EXHIBIT C**) contains approximately 90-95% pro-NGF as disclosed on page 16 of the English translation of the above captioned U.S. Patent Application Serial No. 09/807,096. The crude murine L929 cell lysate that was prepared by the method disclosed in Example 4 of Edwards (absent the infection step) and that is depicted in lane 2 of **EXHIBIT B** is believed to have much less than at least about 90% pro-NGF due to the presence of the contaminating host cell proteins.

14. Various published articles describe the production of crude extracts from eukaryotic and prokaryotic cells that are believed to demonstrate that a protein of interest that is present in a crude extract is not "substantially purified". For example, **EXHIBIT D** is a true and accurate copy of Yokota *et al.* (1999) 121 *Plant Physiology* 525-534 (hereinafter "Yokota"), which describes the production of myosins in cultured tobacco cells. Figure 1 of Yokota depicts SDS-PAGE analysis of clarified lysates of tobacco cells expressing these myosins. Panel A,

lane a of Figure 1 depicts a Coomassie-stained SDS-PAGE gel from an extract that had been clarified by centrifugation at 10,000*g* for 10 minutes, which is believed to be substantially similar to the clarification step disclosed in Example 4 of Edwards. In fact, Yokota further clarified the lysate by centrifuging at 100,000*g* for 30 minutes (*see* page 526, right column, third paragraph), which is believed to be substantially more stringent a clarification step than is disclosed in Edwards. Nonetheless, despite this additional step, it is believed that Figure 1 shows that the proteins of interest were not substantially purified as a result of the centrifugations steps (compare Figure 1, panel A, lanes a and b of **EXHIBIT D**), and that the vast majority of the proteins detected were of host cell origin.

15. Yokota also applied additional purification steps including hydroxylapatite chromatography and an ion exchange column (*see* page 526, right column, fourth paragraph) in order to remove the contaminating host cell proteins to a large extent in order to substantially purify the proteins of interest. In the above captioned U.S. Patent Application Serial No. 09/807,096, proNGF as the protein of interest is isolated in the form of inclusion bodies, which already results in a highly purified protein preparation. *See* Example 2 and Figure 2 of the above captioned U.S. Patent Application Serial No. 09/807,096, reproduced as **EXHIBIT C**. In order to remove any remaining contaminating proteins and to obtain an even higher purity, at least one additional ion exchange purification step as depicted in Example 3d of the above captioned U.S. Patent Application Serial No. 09/807,096 can be performed. HPLC Analysis of the thereby obtained pro-NGF is depicted in Figure 6 of the above captioned U.S. Patent Application Serial No. 09/807,096, and shows a high degree of purity.

16. **EXHIBIT E** is a true and accurate copy of Stauber *et al.* (1988) 263 *Journal of Biological Chemistry* 19098-19104 (hereinafter "Stauber"), in which human Tumor Necrosis Factor-α Receptor (TNFαR) was purified from a human cell line. Stauber discloses that the cells containing the TNFαR were solubilized in a detergent-containing Tris buffer solution, and then clarified by centrifugation at 200,000*g* for one hour. This clarification procedure is believed to be significantly more stringent than that disclosed in Edwards (20-fold higher *g*

forces and for an additional 45 minutes), and yet still resulted in a crude lysate that was significantly contaminated with host cell proteins. This is shown in Figure 5A, in which lane 1 corresponds to the crude lysate and lane 3 corresponds to the immunoaffinity-purified TNFαR. In fact, lane 2 of Figure 5A corresponds to the flow-through from the immunoaffinity column, which includes all of the host proteins. Comparison of lanes 1 and 3 or of lanes 2 and 3 is believed to demonstrate the very significant contamination of the crude lysates with host proteins. Indeed, for the protein of interest, TNFαR, immunoaffinity purifications were also employed (*see* 19100 *et seq.* describing immunoprecipitation and immunoaffinity chromatography).

17. The crude lysate described in Example 4 of Edwards, like the crude lysates disclosed in **EXHIBITS D** and **E** are believed to be characterized by contamination with a very significant level of host cell proteins. As such, it is believed that one of skill in the field would not view the crude lysate described in Example 4 of Edwards as substantially purified for the protein of interest, proNGF.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Susan Lorey

Susan Lorey

28. 09. 2007

Date

Attachments: **Exhibits A-E**